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14. ABSTRACT Toxicity is a major impediment to effective radiation therapy of locally advanced prostate cancer. Work under this award focuses on the potential of a novel class of pharmacological 'radiation protectors' to reduce normal tissue toxicity of radiation therapy. During the first year we screened a series of signal transduction modifiers targeting either canonical NF-κB activation or GSK3 for their potential to protect mice against lethal doses of ionizing radiation. We identified one compound that performed better than the others in preventing catastrophic damage to the epithelial lining of the GI tract and increasing survival of irradiated mice. Importantly, this compound also showed anti-tumor activity against four human prostate cancer cell lines grown as xenotransplants in mice. The compound in question is an anti-inflammatory mediator that inhibits NF-κB activity and other key signaling events of relevance to prostate cancer growth and survival. It exerts these effects by covalently interacting with reactive cysteines on the surface of those proteins. Ongoing work focuses on the characterization of the molecular target spectrum responsible for the differential effects of this drug on normal and tumor tissues.					
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## INTRODUCTION:

Radiation therapy (RT) is a key therapeutic option for prostate cancer, either alone or in combination with hormone therapy. However, the radiation dose that can be safely administered is often lower than the dose considered to be optimal to eradicate tumor cells in the vicinity of the primary lesion, for example pelvic lymph nodes. This is due, in large part, to 'collateral damage' by radiation, i.e. toxicity to the intestine and the bladder. Treatment strategies to escalate the dose of radiation to the pelvic sentinel lymph nodes and/or the primary site, are limited by normal tissue dose constraints that can't be surmounted by IMRT or particle therapy. Hence protection of normal tissue will be a critical requirement for future dose escalation trials in patients with locally advanced disease.

Existing radiation protectors including amifostine (1), sucralfate (2) and mesalazine (3) are of limited utility in protecting the small and large intestines against radiation effects. This motivated us to explore the potential of a novel class of pharmacological 'radiation protectors' to reduce normal tissue toxicity associated with radiation therapy. In preliminary work we and others had identified several 'targeted agents', i.e. small molecule compounds which radioprotect multiple normal tissues including the epithelial lining of the intestinal tract against deleterious effects of high dose radiation. These included pharmacological inhibitors of NF- $\kappa$ B activity and inhibitors of glycogen synthase kinase(GSK)3 that mimic select pro-survival effects of the PI-3-kinase/AKT pathway (Table 1). Of note, the agents under investigation are modulators of signal transduction pathways and, thus, distinct from conventional ROS scavengers or antioxidants such as Amifostine. This is notwithstanding the fact that some of the inhibitors tested (e.g. ethyl pyruvate, CDDO) also exert antioxidant activity

**Table 1: Compounds under investigation.** The compounds indicated below were selected due to their radioprotective properties in zebrafish screens and in mice. All compounds used in zebrafish except CDDO were from Calbiochem/EMD. Note that protection was achieved in zebrafish and mice at roughly equimolar concentrations where data are available in both model systems. Radioprotection of the GI system in zebrafish was selectively tested and observed for EP and CDDO as well as for LiCl, SB216763 and Azakenpaullone. Data were compiled from the following references (4-6). Zebrafish GSK3 inhibitor data from our laboratory are unpublished.

Pathway	Compound	Target(s)	Effective dose (in vitro)
NF- $\kappa$ B	Ethyl Pyruvate (EP)	NF- $\kappa$ Bp65	1 mM
	TX415 (CDDO)	IKK $\beta$ /KEAP1-Nrf2	1 $\mu$ M
GSK3	Lithium Chloride (LiCl)	GS3 (allosteric)	20 mM
	SB216763	GSK3 (ATP competitive)	5 $\mu$ M
	Azakenpaullone	GSK3 (ATP competitive)	0.3 $\mu$ M

## BODY:

In the following we will first focus on radiation protection of mice by the compounds investigated. We will then describe effects of the lead candidate with superior radioprotective properties (TX415, CDDO) on growth and survival of prostate cancer cells in vitro and in vivo.

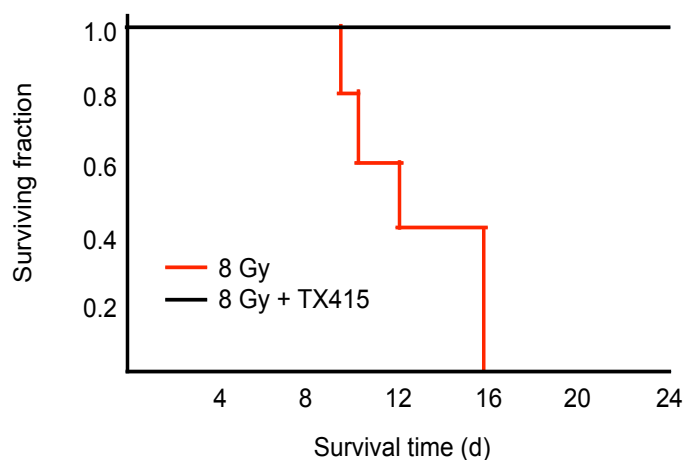
### 1. Radioprotection screen in mice by investigational compounds.

A screen of the compounds listed in Table 1 revealed that TX415 provided radiation protection to mice exposed to lethal doses of radiation (10 Gy; single dose). At this dose level mice die from radiation-induced gastrointestinal syndrome (RIGS). Similar levels of protection were obtained using the GSK3 inhibitor SB216763. Additional experiments pursued under separate funding revealed that TX415 but not SB216763 provided effective protection against and mitigation of hemopoietic syndrome as well. Further, TX415 also provided radiation protection to the skin. Given this broad spectrum of it's radioprotective effects we decided to focus further experimentation on TX415. This decision was also based on the fact that, among all the agents investigated, TX415 (REATA Pharmaceuticals, Inc.) is currently under active development in the oncology space. Finally, TX415 has at least two molecular targets relevant to radioprotection. First, by binding to

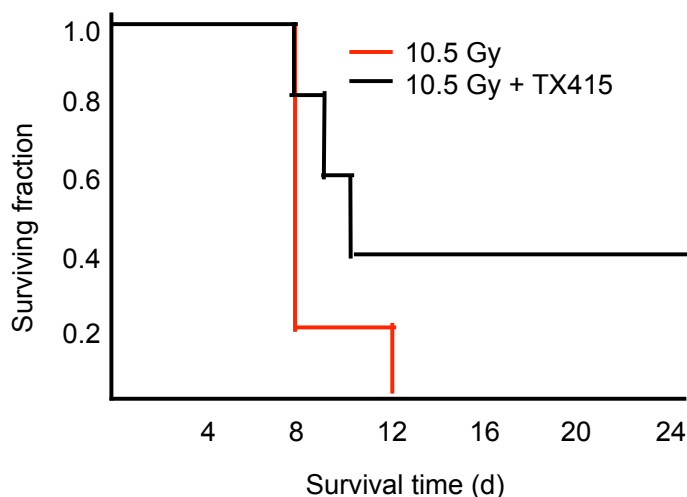
KEAP1, it disrupts degradation of the transcription factor Nrf2 which, in turn, controls expression of a number of proteins with antioxidant properties. Second, by binding to and modifying IKK $\beta$  function it dampens NF- $\kappa$ B – dependent inflammatory changes in cells. In the following we will focus on data obtained with TX415.

## 2. Normal tissue protection by TX415

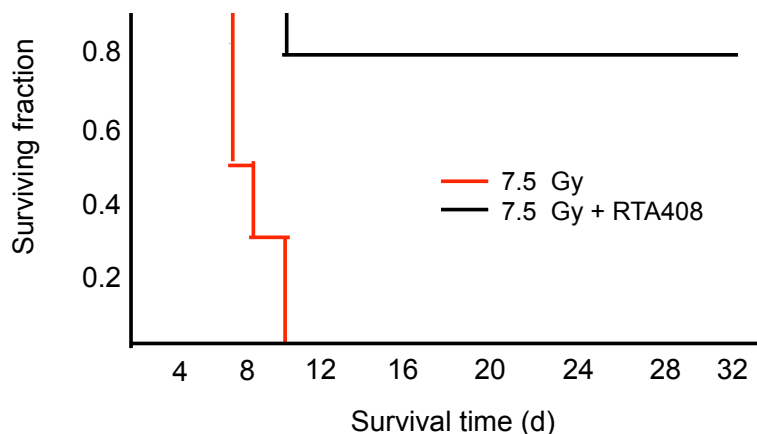
TX415 treatment (17.5 mg/kg, i.p.) of mice 1 day and 1 hour prior to radiation exposure followed by 3 daily doses post IR was fully radioprotective up to 5 weeks for mice that received 8 Gy of IR (Fig. 1) and moderately radioprotective against 10.5 Gy of IR (Fig. 2).



**Figure 1: Radioprotective effects of TX415 – gastrointestinal tract.** C57Bl/6 mice (n=5 per cohort) were administered TX415 (17.5 mg/kg i.p.) or vehicle (DMSO) control 1 day and 1 hour prior to radiation exposure (8 Gy) followed by 3 daily doses post IR. Animals were euthanized at the end of the observation period, when weight loss reached or exceeded 20% of the initial weight, or if they showed signs of severe morbidity.

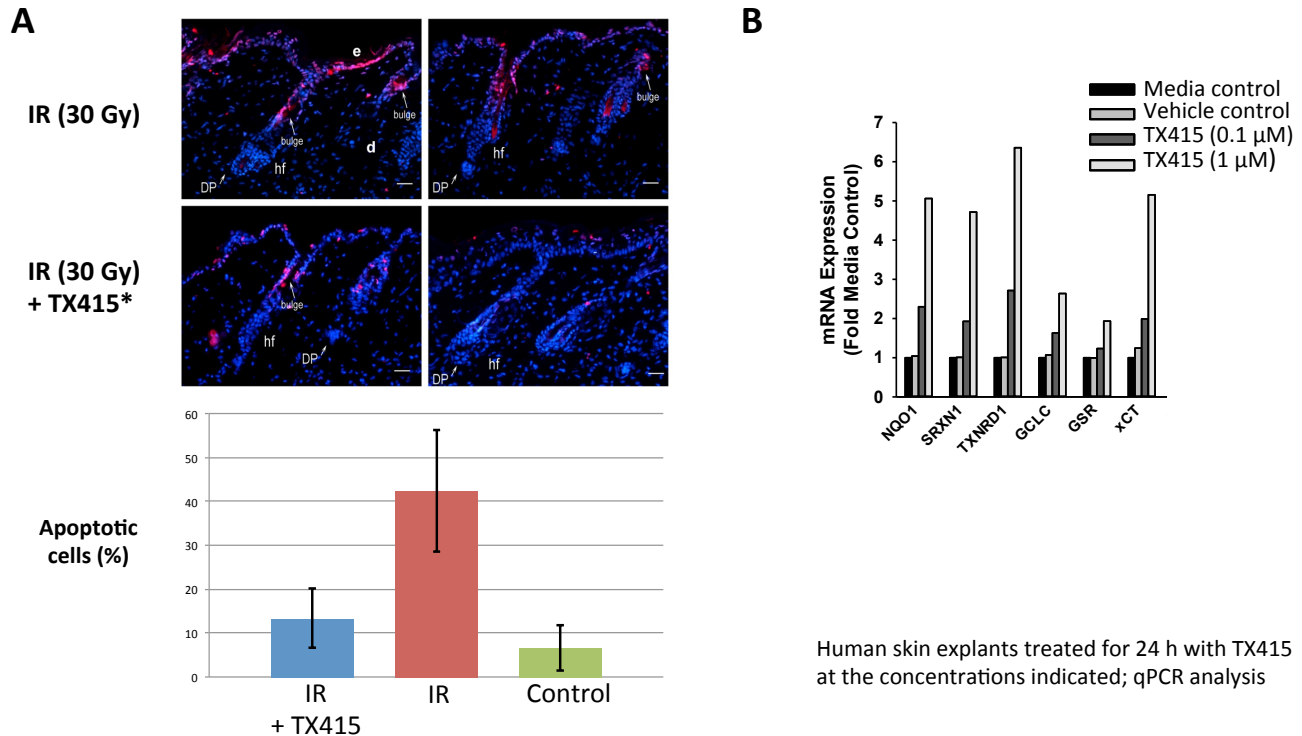


**Figure 2: Radioprotective effects of TX415 – gastrointestinal tract.** C57Bl/6 mice (n=5 per cohort) were administered TX415 (17.5 mg/kg i.p.) or vehicle (DMSO) control 1 day and 1 hour prior to radiation exposure (10.5 Gy) followed by 3 daily doses post IR. Animals were euthanized at the end of the observation period, when weight loss reached or exceeded 20% of the initial weight, or when they showed signs of severe morbidity.



**Figure 3: Radioprotective effects of TX415 – hemopoietic system.** C57Bl/6 mice (n=10 per cohort) were administered TX415 (17.5 mg/kg i.p.) or vehicle (DMSO) control for 3 days post radiation exposure (7.5 Gy). Animals were euthanized at the end of the observation period, when weight loss reached or exceeded 20% of the initial weight, or if they showed signs of severe morbidity. Note long-term survival of TX415-treated mice past 30 days post IR indicative of protection of the hemopoietic system.

In addition, all animals that survived RIGS were also protected against hemopoietic syndrome which occurs at approximately 2-3 weeks after radiation exposure (Figs. 1-3). Finally, we determined effects of TX415 on cutaneous syndrome which occurs after local high-dose (30 Gy) radiation exposure of mice. Figure 4 shows that TX415 markedly reduced the apoptosis incidence in exposed skin consistent with protection of this organ system as well. Interestingly, we were also able to show that, as determined by qPCR, TX415 induced transcript levels of a several genes encoding enzymes with antioxidant properties in human skin explants.

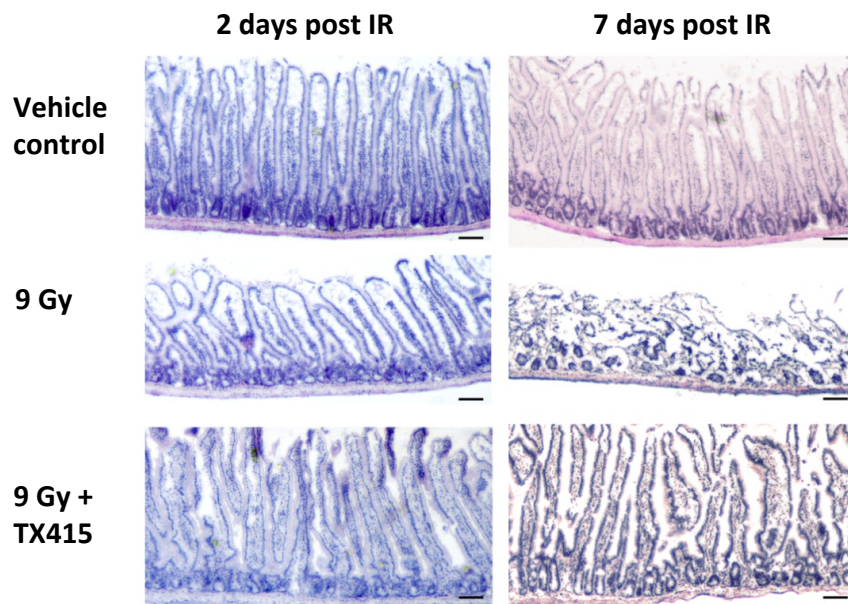


\* 17,5 mg/kg: Protection setting, drug administered 1 d prior, 1 h prior and 1 d post IR; skin collected 2 d post IR

**Figure 4: TX415 attenuates IR-associated apoptosis in murine skin and induces antioxidant enzymes in human skin.** (A) Apoptosis incidence in the epidermis of irradiated mice was determined by in situ TUNEL stain (B) Effects of TX415 on expression of genes encoding antioxidant proteins in human skin explants.

### 3. Mitigation of IR-induced GI structural damage by TX415

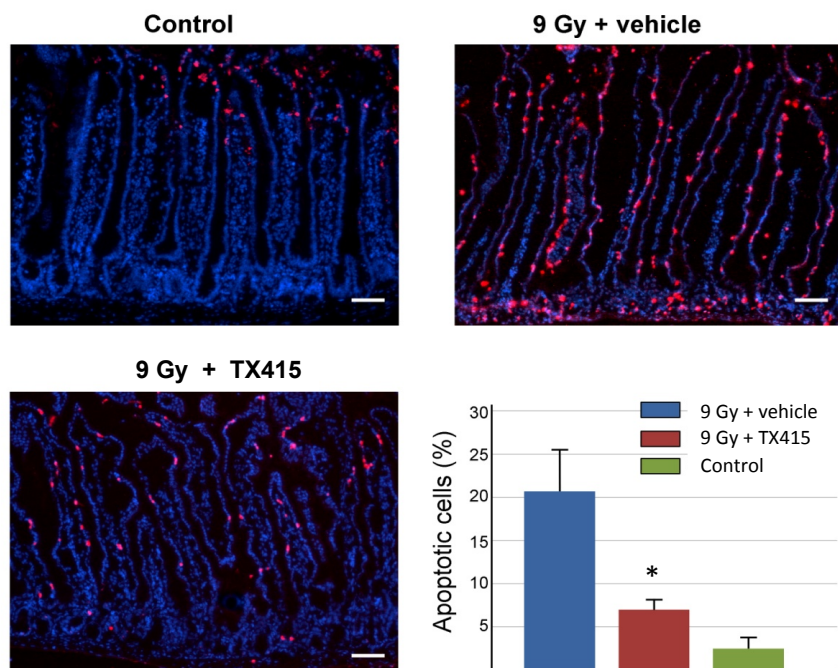
Next , we studies the effects of of TX415 (17.5 mg/kg, i.p.) administered at days 1, 2, and 3 post IR (9 Gy) on structural damage to the lining of the small intestines of irradiated mice (Fig. 5). This analysis revealed almost complete destruction of the epithelial lining of the GI tract in vehicle-treated mice at day 7. Partial preservation of normal crypt and villus architecture is apparent in TX415 treated animals. Control unirradiated tissue is shown for comparison to illustrate healthy small intestine villi structure and organization.



**Figure 5: TX415 preserves the integrity of the gastrointestinal tract of irradiated mice.** C57Bl/6 mice (n=3 per cohort) were administered TX415 (17.5 mg/kg i.p.) or vehicle (DMSO) control 1, 2, and 3 days after IR exposure (9 Gy). Hematoxylin and Eosin stained sections of the small intestine from representative animals at days 2 and 7 post-IR are shown. Scale bar – 100  $\mu$ m. Unirradiated mice served as controls.

#### 4. Mitigation of IR-induced apoptosis in the small intestine by TX415

TX415 treatment in the radiation mitigation setting (as described in the preceding paragraph) further reduced the number of apoptotic cells in the small intestine of IR exposed animals by more than 60% (Fig. 6). The protection of cells within the crypts is considered particularly relevant as epithelial stem cells reside in this tissue compartment.



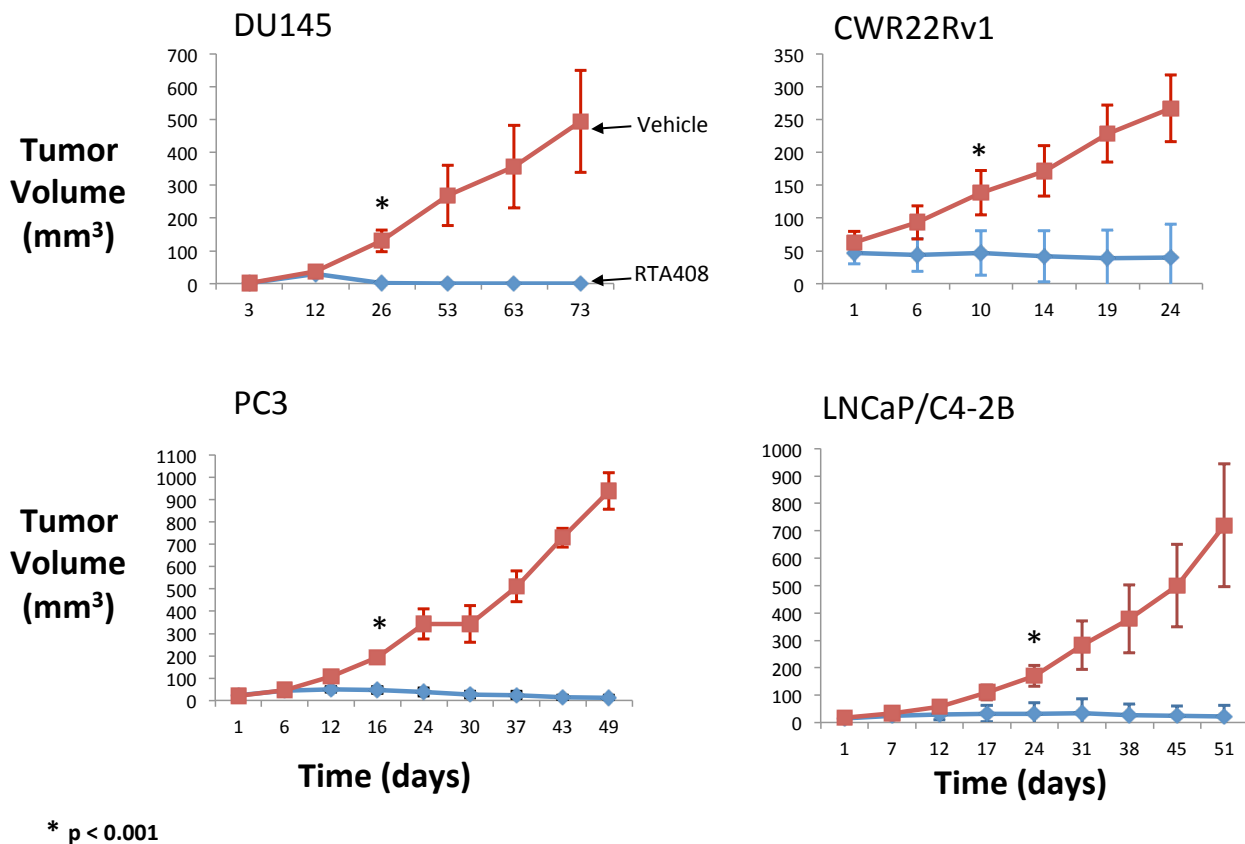
**Figure 6: Mitigation of IR-induced apoptosis in the small intestine by TX415.** C57Bl/6 mice (n=3 per cohort) were administered TX415 (17.5 mg/kg i.p. in DMSO) or vehicle control 1, 2, and 3 days after IR exposure (9 Gy). Sections of the small intestine were processed for TUNEL staining to compare the frequency of apoptotic cells. Red – TUNEL positive apoptotic cells, Blue – DAPI nuclear counterstaining. Scale Bar – 100  $\mu$ m. Quantitation of apoptosis (mean + SD) was done using ImagePro software on 10 different and independent microscopic fields for each treatment condition. Apoptosis incidence was statistically significantly ( $p < 0.05$ ) reduced in TX415 treated mice as compared to vehicle-treated mice.



Collectively, these results support the notion that TX415 provides multi-organ protection against deleterious effects of high dose radiation exposure. In human skin, this effect was associated with induction of enzymes with antioxidant properties. Further mechanistic studies are necessary to pinpoint the pathway(s) responsible for the broad-spectrum cytoprotective effects of TX415 and will be pursued during years 2 and 3 of this award.

## 5. Tumor growth inhibition by TX415

Next, we addressed the question whether cytoprotection by TX415 extended to human prostate cancer cells. This essential part of this study was done using two hormone-independent established prostate cancer cell lines (PC3 and DU145) and two cell lines that express the androgen receptor (CWR22Rv1 and LNCaP/C4-2B). All four cell lines were grown as xenografts in nude mice for varying time periods and treated with TX415 (17.5 mg/kg; i.p. administration). Strong anti-tumor effects of TX415 were observed for all four cell lines tested (Fig. 7).



**Figure 7: TX415 inhibits growth of prostate cancer xenografts.** Drug was administered 3 x per week at 17.5 mg/kg for the duration of the experiments. The x-axis shows time on treatment which varied for the different tumor cell lines. In all experiments significant tumor growth inhibition was achieved.

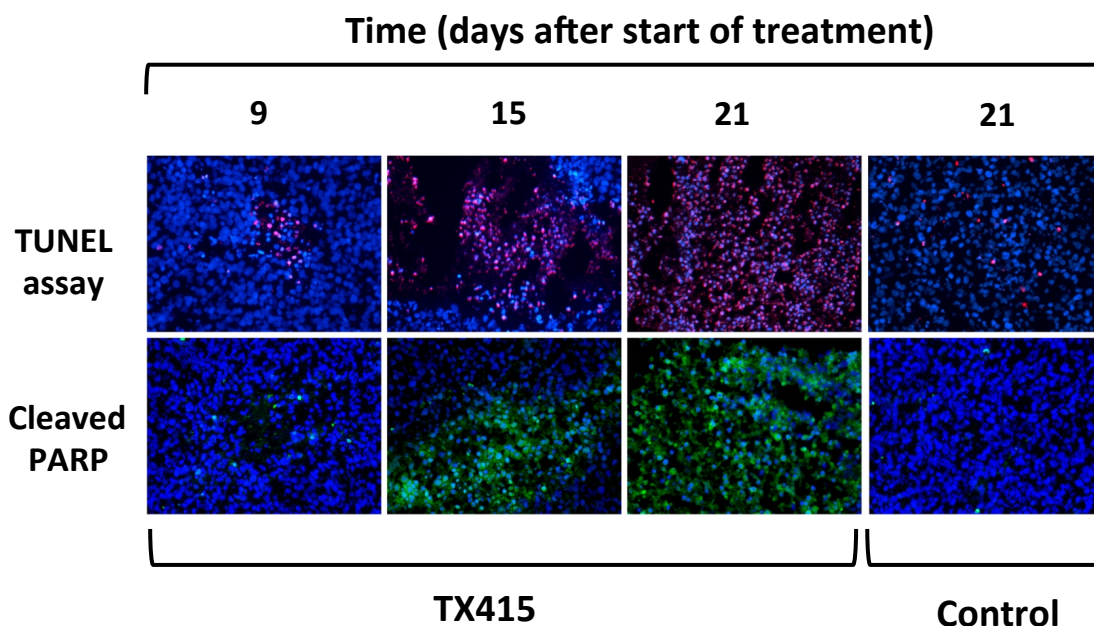
In the case of PC3 cells tumor growth inhibition was associated with extensive apoptosis as determined by in situ TUNEL assay and detection of cleaved PARP (Fig. 8). Note that these effects were delayed and occurred at approximately 2 weeks after treatment was commenced. This result is consistent with the tumor growth kinetics observed in vivo (Fig. 9) as PC3 tumors continued to grow during the first 10 days of drug administration followed by a marked decreases in tumor size. Similar delayed effects on tumor growth were observed DU145 cells as well. The reason for the delayed kinetics of tumor growth inhibition and apoptosis induction by TX415 are presently unclear and under investigation.

These results are consistent with the notion that TX415 exerts dual and opposite effects on normal and tumor tissues. It appears to protect normal tissues including the epithelial lining of the gastrointestinal tract against

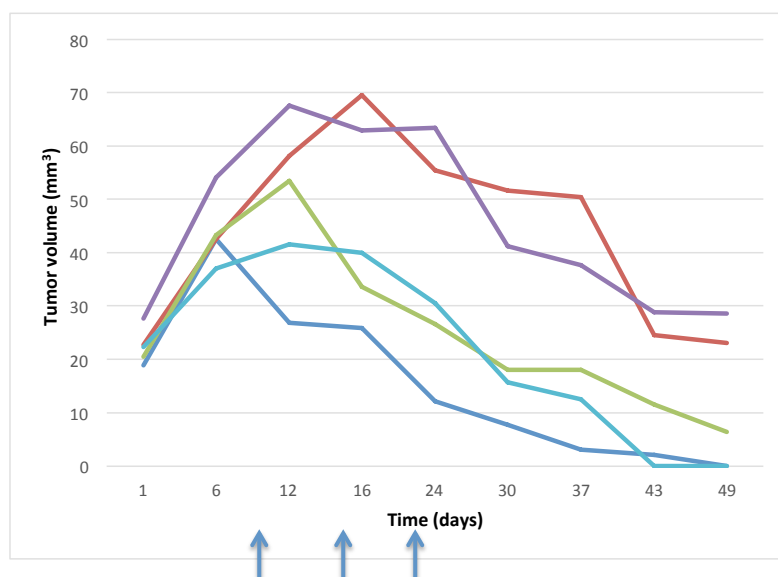


deleterious effect of radiation while inhibiting the growth of prostate cancer cells in vivo. These results support further testing of this experimental compound as a selective radiation protector in humans. They also provide a strong rationale to identify the molecular pathways responsible for the divergent effects of TX415 on normal and malignant tissues. This will be a major focus of work going forward.

Of interest, increased apoptosis in xenotransplants became prevalent only 9-15 days after drug administration commenced. In addition, during the first 10 days of drug administration tumor volumes increased and only began to decrease afterwards. These results indicate a delayed effect of TX415 on prostate cancer grown in vivo. Future work will focus on potential mechanism underlying the protracted kinetics of TX415 anti-tumor effects.



**Figure 8: TX415 induces apoptosis in PC3 xenotransplants in vivo.** PC3 cell were inoculated into nu/nu mice and tumors were established. Mice harboring established tumors were treated with RTA 408 or DMSO (control). At the time points indicated, tumor tissues were collected and examined for the induction of apoptosis by in situ cell (TUNEL) assay detecting nicked DNA and by indirect immunofluorescent detection of the cleaved PARP.



**Figure 9: Delayed effects of TX415 on growth of PC3 xenotransplants in mice.** Each line represents the tumor volume over time of an individual xenotransplant (n=5). Blue arrows abutting the x-axis indicate time points at which sentinel mice were sacrificed and small intestine samples processed for in situ analysis of apoptosis (see Fig. 8).

Finally, we have performed exploratory experiments combining TX415 with IR to test whether TX415 radioprotects human prostate cancer xenotransplants. In two independent experiments we found no evidence for radioprotection. Future work will vary the radiation and drug doses to evaluate whether the two treatment modalities exert additive anti-tumor effects.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- Screened the compounds listed in the original proposal for radioprotective properties in whole organisms.
- Identified a lead candidate (TX415) for superior radioprotection of normal tissues (gastrointestinal system, skin) of relevance to prostate cancer therapy.
- Characterized cytoprotective and anti-apoptotic properties of TX415 on normal GI stem cell niche.
- Established anti-tumor activity of TX415 on four prostate cancer cell lines.
- Identified apoptosis induction associated with anti-tumor effects of TX415 in vivo.
- Initiated experiments to assess combined effects of TX415 and radiation on human prostate cancer growth in mice.
- Initiated pathway analyses to discern why the same compound selectively radioprotects normal but not tumor tissues.

## **REPORTABLE OUTCOMES:**

- Invited oral presentation of results at the 20<sup>th</sup> Annual Prostate Cancer Foundation Retreat in October 2013.
- Developed human prostate cancer cell lines stably expressing reporter constructs for in vivo imaging and for NF- $\kappa$ B activity.
- Additional funding secured from REATA Pharmaceuticals to molecularly define radioprotective properties of TX415.
- Additional funding obtained to conduct a two-year pilot project to explore TX415 as a mitigator of radiation damage in the context of the CMCR (Center of Countermeasures against Radiation; NIH NIAID) at Einstein Medical Center, Bronx, NY (PI: Dr. C. Guha).

## **CONCLUSION:**

Treatment of mice with ionizing radiation (IR) leads to intestinal cell death, structural GI damage, weight loss, and mortality. We have identified a compound (the triterpenoid TX415) that alleviates all of the criteria of radiation toxicity in mice. Treatment of mice with TX415 (17.5 mg/kg) both before or after IR exposure (dose range 7.5 to 10.5 Gy) provided a striking survival advantage and protection of multiple organs including the gastrointestinal system and the skin. Overall, TX415 effects were robust, extended to several organ system and were superior to those observed with the other compounds investigated. The radioprotective properties of TX415 are contrasted by uniform and strong anti-tumor effects of TX415 on the growth of human prostate cancer cells in nude mice. The anti-tumor effects are accompanied by delayed induction of apoptosis in tumor tissues in situ. In summary, our screen has identified a compound with opposite effects on normal mouse and human xenotransplant tumor tissues. This compound is well suited for clinical studies to evaluate normal tissue protection in the radiation setting. The results obtained thus far also call for a systematic investigation of molecular mechanisms responsible for the remarkable opposite effects of TX415 on normal irradiated tissues and tumor xenotransplants.

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